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(71) Applicant (*for all designated States except US*): NICKERSON BIOCEM LIMITED [GB/GB]; Cambridge Science Park, Milton Road, Cambridge CB4 4GZ (GB).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): TAYLOR, Mark, Andrew [GB/GB]; 9 Newhall Gardens, Dundee DD2 1TW (GB). DAVIES, Howard, Vivian [GB/GB]; 2 Station Road, Invergowrie, Dundee DD2 5DU (GB).

(74) Agents: CORNISH, Kristina, Victoria et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).

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(57) Abstract

The present invention provides recombinant or isolated nucleic acid encoding an α-glucosidase enzyme, especially those nucleic acid sequences encoding a plant α-glucosidase enzyme. Antisense nucleic acid is also provided, as well as the use of both the isolated or recombinant sequences and the antisense sequences. Uses of the invention include enhancing and reducing expression of α-glucosidases and the provision of novel starches.

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POTATO ALPHA-GLUCOSIDASE GENE

This invention relates to the fields of plant biotechnology and plant genetic engineering. In particular, it relates to the production of novel starches by plants and to the manipulation of starch-sugar conversions in transgenic plants by changing the cellular activity of the α-glucosidase enzyme. More specifically, it relates to nucleic acid sequences encoding α-glucosidase enzymes and the potential use of such sequences when expressed in plants.

It is known that the mobilisation of starch by hydrolysis in plants involves the enzymes α-amylases, β-amylase, debranching enzyme and α-glucosidase. However, the precise roles of the of starch degrading enzymes and their mechanisms of action remain unclear. One view is that α-amylases are the only enzymes that use native starch as a substrate for hydrolysis. Accordingly, the role of ß-amylase, debranching enzyme and α-glucosidase is to hydrolyse the dextrins released by α-amylase activity on the granule (reviewed by Beck and Zeigler, 1989). Conversely, some evidence suggests that α-amylase cannot act on starch grains alone and requires other factors for starch degradation (Stamberg and Bailey, 1939). A good candidate for this α-amylase activating factor is α-glucosidase (Schwimmer, 1945). In fact some α-glucosidases have been shown to catalyse the hydrolysis of soluble starch, although at very low rates (Yamasaki and Konno, 1985). In germinating barley there was a high degree of synergistic starch hydrolysis when α-amylase and α-glucosidase were incubated with starch granules (Sun and Henson, 1990). More recently, it has been demonstrated that a pea chloroplastic α-glucosidase is capable of initiating chloroplastic starch grain degradation (Sun *et al.*, 1995). This may cause starch grains to be more susceptible to further hydrolysis by

other enzymes. Thus, it appears that the importance of α-glucosidases in starch hydrolysis may have been underestimated.

α-Glucosidases catalyse the hydrolysis or transfer of the terminal α-D-glucosyl residues of α-D-glucosidically linked carbohydrates. A general feature is that, unlike other starch degrading enzymes, a broad range of compounds are substrates for these enzymes. Generally, as well as catalysing the hydrolysis of maltose, they will also use oligosaccharides (maltodextrins) and polysaccharides (amylose, amylopectin and glycogen) as substrates (Yoshikawa *et al.*, 1994). Additionally, most α-glucosidases will hydrolyse a range of glucobioses containing α-1,2, α-1,3, α-1,4 and α-1,6 bonds. This may be particularly important if all these type of linkages are present *in vivo*, as other starch degrading enzymes such as α-amylase cannot catalyse the breakdown of α-1,2 and α-1,3 bonds (Sun *et al.*, 1995). Some evidence suggests that these types of linkages do exist in starch grains (Abdel-Akher, M. *et al.*, 1952, Wolfrom and Thompson, 1956). α-Glucosidases also catalyse transglycosylation reactions that may be important *in vivo*. For example, isomaltose, maltotriose and panose can be synthesised from maltose (Yamasaki and Suzuki, 1980, Yamasaki and Konno, 1985) and kojibiose, nigerose and maltose can be synthesised from soluble starch (Chiba, 1988).

It is well known that plants with novel characteristics can be produced by the expression of gene sequences introduced by transformation procedures such as *Agrobacterium*-mediated or vector mediated transformation methods or physical transformation methods such as

biolistics, chemical or electrical transfection or micro-injection, introducing genes or DNA sequences (Draper *et al.*, 1988). Despite the widespread occurrence of α-glucosidases in higher plants and their importance in starch and carbohydrate metabolism, no α-glucosidase genes from plants have been characterised.

This has the disadvantage of preventing a transgenic approach for the manipulation of the expression of α-glucosidase genes.

Accordingly, a first aspect of the present invention provides a recombinant or isolated nucleic acid encoding an α-glucosidase enzyme, preferably a plant α-glucosidase enzyme. Nucleic acid according to the present invention is preferably DNA but also includes cDNA and RNA. The α-glucosidase enzyme of the present invention includes sequences which are preferably obtainable from plants or microbes including, in particular, the plants: potato, pea, maize, wheat, rice, barley, sweet potato, cassava or yam, amongst other species.

The present invention also includes, according to all aspects, mutations and fragments of nucleic acid sequences encoding α-glucosidases (preferably α-glucosidases obtainable from plants) and mutations and fragments of amino acid sequences of those α-glucosidase enzymes. The present invention particularly includes nucleic acid and amino acid sequences of α-glucosidase which are obtainable from the potato cultivars Record, Desiree, Binje or Russet Burbank as well as mutants and fragments thereof.

Fragments of nucleic acid according to the present

invention include 9 or more, preferably 12 or more, preferably 15 or more, preferably 18 or more bases and the corresponding number of amino acids. When used to describe the invention, the phrase 'a part of' means any size fragment thereof.

The recombinant or isolated nucleic acid according to the first aspect of the invention preferably encodes for an enzyme having the activity of a 105.4 kD α-glucosidase enzyme of potato, especially the potato cultivar Record.

The recombinant or isolated nucleic acid nucleic according to the first aspect of the invention most preferably encodes at least part of (ie. a fragment of) the amino acid sequence as shown in Figure 2. The recombinant or isolated nucleic acid preferably encodes an amino acid sequence having more than 29% identity with the sequence shown in Figure 2, more preferably 40% identity, even more preferably 60% identity.

The recombinant or isolated nucleic acid may have a coding sequence operatively linked to a promoter. The promoter may be constitutive, for example the well known CaMV35S promoter or inducible, for example, the GAL/GAL10 promoter. The promoter may also be tissue-specific, for example, the tuber-specific promoter GBSSI, the tuber-specific patatin I promoter (Kim et al), the tomato fruit-specific E8 promoter (Lincoln et al) and the promoter of the small subunit of the ribulose-1,5-biphosphate carboxylase gene (Coruzzi et al).

According to a second aspect of the invention, there is provided a recombinant or isolated nucleic acid comprising a promoter which naturally drives expression

of a nucleic acid sequence encoding an α-glucosidase enzyme, preferably a plant α-glucosidase enzyme. The coding sequence for the α-glucosidase is preferably as described above, according to the first aspect of the invention.

The recombinant or isolated nucleic acid according to the first and second aspects of the invention, when expressed, may result in enhanced starch and/or other carbohydrate breakdown, preferably in a plant. It may also result in novel starch structures. The enhanced breakdown is a result of increased expression of the α-glucosidase enzyme, usually compared to endogenous levels. According to promoters used, for example, a tissue specific promoter, the increased levels of α-glucosidase can be limited to desired tissues, such as plant storage organs (for example, potato tubers).

The recombinant or isolated nucleic acid of the first and second aspects of the invention may include a signal sequence in translational fusion with the α-glucosidase coding sequence. Such a sequence may enable the expression of an introduced α-glucosidase sequence specifically in a cellular organelle. For example, the GBSSI signal sequence targets expression to the plastid in potato tubers (Visser et al 1989).

According to a third aspect of the invention there is provided antisense nucleic acid to nucleic acid encoding α-glucosidases of the present invention. Antisense nucleic acid is well understood in the art. Thus the present invention provides antisense nucleic acid which comprises a transcribable strand of nucleic acid, the transcribed nucleic acid being complementary to at

least part of a strand of nucleic acid which is transcribed from a nucleic acid sequence encoding a α-glucosidase enzyme. Preferably, the nucleic acid sequence encoding the α-glucosidase enzyme is obtainable from a plant, but it may also be obtainable from other sources, such as microbial sources (eg. yeast) The transcribable nucleic acid of the antisense and/or encoding the α-glucosidase may be recombinant. The nucleic acid encoding the α-glucosidase may be a 'natural' (endogenous) nucleic acid sequence (which may also be recombinant) or may be a modified sequence. The nucleic acid encoding the plant α-glucosidase enzyme may be obtainable from a potato, in particular, the potato cultivar Record. The nucleic acid encoding the plant α-glucosidase enzyme may preferably encode for an enzyme having the activity of a 105.4 kD α-glucosidase enzyme of potato.

The antisense nucleic acid is advantageously complementary to part of a nucleic acid sequence encoding for the amino acid sequence shown in Figure 2. Preferably the antisense nucleic acid is complementary in the same level of identity as described hereinbefore for the recombinant or isolated nucleic acid. In this way, the transcribable strand of nucleic acid from the antisense nucleic acid will be complementary to at least part of the strand of the nucleic acid which is transcribed from the nucleic acid encoding a plant α-glucosidase enzyme.

The antisense nucleic acid may be operatively linked to a promoter, as described according to the first aspect of the invention.

According to a fourth aspect of the invention there is provided nucleic acid capable of disrupting the proper expression of an α-glucosidase gene, preferably a plant α-glucosidase gene, most preferably an α-glucosidase gene of potato, pea, maize, wheat, rice, barley, sweet potato, cassava or yam. The disruption may be by any recombinant nucleic acid technology, including those well known in the art. The nucleic acid capable of the most effective disruption, disrupts proper expression of a gene encoding an α-glucosidase enzyme having the activity of a 105.4 kD α-glucosidase enzyme of potato. Nucleic acid capable of disrupting the proper expression of an α-glucosidase gene, according to the invention, may comprise nucleic acid obtainable from a source other than a plant eg. a microbial source, in particular a yeast.

The nucleic acid according to any aspect of the invention may comprise a 5' transcription regulation sequence such as the 5' transcription regulation sequence derived from the Cauliflower Mosaic Virus 35S gene, GBSSI, patatin, E8 and the promoter of the RUBISCO small subunit gene.

Nucleic acid, according to any aspect of the invention may comprise part of a vector. Suitable vectors are well known in the art and include cloning vectors such as lambda Zap II (Stratagene) and expression vectors such as pYES2 (Invitrogen). Preferably, the vector comprises one or more selectable markers such as antibiotic resistance.

A construct, comprising nucleic acid according to any one of the aspects of the invention, and including the vectors according to the invention can be introduced into a host cell by transfection or transformation. Such methods are well known in the art. The host cell can

than be used to express and monitor expression of α-glucosidase activity or can be used to clone plants, microbes and/or parts of plants, including tissue culture.

Preferably, a construct comprising a promoter operatively linked to a nucleic acid sequence, encoding an α-glucosidase enzyme can be transformed into a plant or other organism, such as a microbe, eg. yeast. Suitable plants include potato, pea, maize, wheat, rice, barley, sweet potato, cassava or yam amongst others. Suitable transformation techniques are well known in the art as described above. The transformation results in plants, or other organisms, at least some of the cells of which contain a foreign chimeric nucleic acid sequence composed of a promoter operatively linked to nucleic acid encoding an α-glucosidase enzyme. These constructs can include nucleic acid encoding any α-glucosidase (eg. plant, or microbial) for introduction into plant, microbial or animal cells.

In this way, the nucleic acid of the present invention can be used to modify starch and/or other carbohydrate breakdown in material, in particular in plant material. Clearly, the most effective use of such systems are when the promoter sequence drives preferential expression of the nucleic acid in plant storage organs and/or seeds. As a result, the rate of starch and/or other carbohydrate breakdown in plant materials can be modified as well as the potential to produce novel starches and/or other carbohydrates in plants, preferably plant storage organs.

The nucleic acid sequences, constructs, vectors etc., according to the first aspect of the invention, can be

used to obtain enhanced α-glucosidase activity in plant or other organisms material/tissues. A consequence of this is a more efficient breakdown of starch and/or other carbohydrates and the opportunity to product novel starches and/or other carbohydrates. This is a benefit to processes which lead to the production of glucose and/or other starch derived products such as maltose and maltose oligosaccaharides. These includes the malting and brewing process in which the efficiency of starch conversion to glucose will be increased, leading to increased spirit yield. Additionally, increased starch breakdown will increase the glucose content of plant tissues and will consequently enhance the flavour characteristics of plant tissues (for example the fruit of tomato, strawberry, raspberry, blackcurrent).

Furthermore, the nucleic acid sequences, constructs, vectors, etc., according to the third and fourth aspects of the invention can be used to obtain reduced α-glucosidase activity in plant or microbial material. Inhibition of α-glucosidase activity by transformation of plant tissue with constructs containing the potato α-glucosidase sequence in antisense orientation will result in inhibition of starch breakdown in seeds and other storage organs. This will be of use in developing tissues, in storage tissue and in germinating systems. Applications also include inhibition of pre-sprouting in cereals, sprout control in potato, minimising starch and dry weight losses in storage and during cooking or processing. A potential outcome is restricted starch turnover rates and a subsequent increase in the amount of starch deposited.

The nucleic acid sequences of the present invention can

be used as hybridisation probes to clone α-glucosidase genes from other sources and species, for example microbes and pea, maize, wheat, rice, barley, sweet potato, cassava and yam (respectively).

A further aspect of the present invention also provides for novel starch structures, in particular in plant materials. The novel starches are a result of the α-glucosidase activity at differing levels and/or from differing sources. The novel starch structures may have a changed branched structure and/or a changed branch length.

The invention is illustrated by the accompanying drawings in which:

Figure 1. shows the nucleotide sequence of the coding strand of the potato cultivar Record cDNA that encodes α-glucosidase.

Figure 2. shows the deduced amino acid sequence from the cDNA sequence in Figure 1.

Figure 3. shows a comparison of the deduced amino acid sequence of the potato α-glucosidase (labelled pot) with that of the human lysosomal sequence (labelled hum). The comparison was carried out using the default parameters of the GAP programme of the GCG package.

Figure 4. shows growth of yeast strains. (o) non-transformed *ABYSMAL81* with 2% glucose as carbon source, (●) *ABYSMAL81* transformed with *pMAL1YES2* with 0.1% galactose and 2% maltose as carbon source, (□) *ABYSMAL81* with 0.1% galactose and 2% maltose as carbon source and

(■) *ABYSMAL81* transformed with *pYES2* with 0.1% galactose and 2% maltose as the carbon source.

Figure 5. shows α-glucosidase activity in crude extracts of *ABYSMAL81* transformed with *pMAL1YES2* measured in the pH range 3-9.

The invention is illustrated by the following non-limiting examples.

Example 1

Isolation and sequence analysis of a potato α-glucosidase clone.

An *Arabidopsis* expressed sequence tag (EST) (European Molecular Biology Laboratory (EMBL) accession number; t04464.em_est) with sequence similarity to the human lysosomal α-glucosidase gene (Hoefsloot *et al.*, 1988) was used as a probe to obtain the corresponding potato cDNA (*pMAL1*). The EST was generated in an *Arabidopsis* expressed sequence project and can be accessed as described in Newman *et al.*, 1994. A potato cDNA (cultivar Record) library constructed in Zap II (Stratagene) from tuberising stolon tip mRNA (Taylor *et al.*, 1992) was screened by standard techniques using this EST as a probe. Following *in vivo* excision and sub-cloning, DNA sequence of the *pMAL1* clone was obtained for both strands using cycle sequencing (DyeDeoxy Terminator kit, Perkin Elmer) and a 373 automated DNA sequencer (Applied Biosystems). DNA sequence analysis was carried out using software available on the SEQNET Computational Molecular Biology Facility at SERC Daresbury Laboratory UK. The size of the insert in the *pMAL1* was 2992bp (Fig. 1). An open reading frame from the nucleotide in position 62 to

a termination codon at position 2819 was identified. The sequence around the putative ATG initiation codon (A at position 51) is AACCATGA and shares some similarity with the preferred context for higher eukaryotes (CACCATGG, Kozak, 1987). The polypeptide encoded by this ORF has a molecular weight of 105.4 kD and an isoelectric point of 5.75. The deduced amino acid sequence of the *pMAL1* open reading frame was compared with sequences in the EMBL database. The most significant matches were with members of family 31 of glucosyl transferases which includes α-glucosidases from human (Hoefsloot *et al.*, 1988) and *Candida tsukubaenis* (Kinsella *et al.*, 1991). The most similar full length sequence was that of a human lysosomal α-glucosidase (Hoefsloot *et al.*, 1988, Fig 2). Overall the sequences share 50% similarity and 29% identity. The two sequences were less similar in the amino-terminal portion (41% similarity, 16% identity over the first 280 amino acids) however over the remaining portion of the sequence there is 53% similarity and 34% identity. Clusters of identical amino acids occur throughout this region. One of the most highly conserved regions is adjacent to the aspartic acid residue at amino acid 516 of the potato sequence. Regions highly similar to this are at the active site of rabbit isomaltase and sucrase (Hunziker et al 1986). The *pMAL1* ORF was also compared with the deduced amino acid sequences of ESTs from *Arabidopsis*, rice and *C.elegans* that show similarity to α-glucosidases. The *pMAL1* ORF shared 77%, 76% and 58% identity, respectively with these sequences (not shown).

Example 2

Complementation of a yeast α-glucosidase mutant.

To confirm that the *pMAL1* clone did encode a functional α-glucosidase gene, its ability to complement an α-glucosidase-negative mutant strain of *Saccharomyces cerevisiae* was investigated. Such a mutant strain of *Saccharomyces cerevisiae* designated *ABYSMAL81* (Kopetzki *et al.*,1989) has the genotype *ura3-52mal1S-^pra1prb1prc1cps1lys*. This mutant was used in transformation studies using standard yeast culture and manipulation methods as described by Rose *et al.*, 1990. The open reading frame encoding the potato α-glucosidase was cloned into the *Hind* III site of the yeast expression vector *pYES2* (Invitrogen) to generate the construct *pMAL1YES2* using standard techniques. The coding region of the cDNA clone was amplified using the Expand High Fidelity PCR system (Boehringer Mannheim) and the sequence of the PCR product was verified. PCR primers were designed to incorporate *Hind* III sites at the 5′ and 3′ termini of the fragment and the initiation codon was preceded by an AT rich sequence (TTAAA) in order to enhance efficient translation initiation (Romanos *et al.*, 1995). The sequence of the primers used for building the yeast expression construct containing the amplified potato α-glucosidase sequence were:

5′ primer: cgaagcttaaaatgagagctccactactc
3′ primer: cccaagcttgaatcgaccaatcatc

The lithium acetate transformation method (Rose *et al.*, 1990) was used to introduce the plasmids *pYES2*and *pMAL1YES2* independently into the yeast strain *ABYSMAL81*. Transformants were selected for uracil auxotrophy by standard procedures. Expression of the *MAL1* sequence in the *pMAL1YES2* construct was under the control of the *GAL1* portion of the *GAL1/GAL10* promoter which is inactive in

the absence of galactose, induced a 1000 fold in its presence and is subject to glucose repression (Johnston, 1987). Growth of *pMAL1YES2* transformants was observed in the presence of 0.1% galactose and 2% maltose at a rate similar to that of the non-transformed mutant and vector transformed mutant growing on 2% glucose as the carbon source (Fig. 4). It was confirmed that the non-transformed mutant and vector transformed mutants were incapable of growth on this maltose/galactose medium and the transformant required the addition of galactose for growth. Presumably the level of galactose in the medium (0.1%) was too low to support growth of the mutant but was sufficient to induce expression from the *GAL1* promoter.

α-Glucosidase activity in crude yeast extracts was determined in the transformed and non-transformed mutant strains. Overnight cultures of yeast (50 ml) were harvested by centrifugation (2000g, 5 min) and the cell pellet resuspended in 0.5 ml of 10 mM sodium phospate buffer, pH 6.5. Cells were broken by sonication using a Misonix Ultrasonic Cell Disruptor (Misonix Inc) and cell debris was then sedimented by centrifugation (16000g, 20 min). Small molecules were removed from the supernatant by gel filtration using a PD-10 Sephadex G-25 M column (Pharmacia). α-Glucosidase activity was determined using a range of maltodextrins, amylopectin and boiled soluble starch as substrates. The reaction mixture (100 L) contained 50mM maltodextrin or 2% (w/v) amylopectin or boiled soluble potato starch substrate in sodium phosphate buffer pH 6.5, and 50 μL of crude extract and was incubated for 1 hour at 30°C. The reaction was terminated by boiling for 5 minutes and the amount of glucose released determined by the reduction of NAD^+ in

the coupled reactions of hexokinase and glucose-6-phosphate dehydrogenase in the presence of ATP and NAD^+ in a microtiter plate assay (Viola and Davies, 1992). Significant activity was detected following transformation of the *ABYSMAL81* mutant with *pMAL1YES2* and galactose induction. No activity could be detected in the non-transformed or *pYES2* transformed *ABYSMAL81* strain (Table 1). The optimum pH for α-glucosidase activity was 6.5 (Fig.5).

The rate of hydrolysis of a range of maltodextrins, amylopectin, and boiled starch was also investigated (Table 1). The greatest activity was observed when maltotetraose was the substrate (151% of the maltose rate). Very little activity (5% of the maltose rate) was detected when amylopectin or boiled soluble starch were used as substrates although this was significantly greater than in the non-transformed mutant.

Table 1. α-Glucosidase activity in crude extracts of *ABYSMAL81* transformed with *pMAL1YES2*. The 100% maltose rate was 3.25 nmoles glucose/ g protein / hr.

Substrate	**α-glucosidase activity. (percentage of maltose rate)**
maltose	100
maltotriose	142
maltotetraose	151
maltopentaose	117
maltohexaose	68
maltoheptaose	50
amylopectin	5
boiled starch	5

Example 3

Transformation studies.

Preliminary transformation studies were carried out using constructs containing the potato *MAL1* sequence. Two constructs were built as described below. An antisense construct containing the entire *MAL1* coding sequence in reverse orientation under the control of two copies of the constitutive CaMV 35S promoter and containing the nos terminator was cloned into the plant transformation vector pBIN19 (Bevan, 1984). This vector contained a kanamycin selection marker. A sense construct was built as above except the entire *MAL1* coding region was cloned in the sense orientation. 59 independent (determined by Southern analysis (Sambrook *et al.*, 1989)) transgenic potato antisense lines and 45 sense lines were generated in the potato cultivar Desiree following standard transformation protocols (Kumar, 1995).

Example 4

α-Glucosidase activity measurements.

α-Glucosidase activity measurements were carried out (Cochrane *et al.*, 1991) on tuber and leaf samples from the *MAL1* antisense plants in Example 3. In tubers from these transgenic lines, a small (20%) but consistent decrease in activity was detected on either a protein or fresh weight basis in three lines. In leaf samples from the antisense lines a significant decrease in activity was measured, in some lines the activity was approximately 50% that observed in vector-only transformed leaves. Other lines showed a decrease in activity of up to 50%.

Example 5

Tuber sugar content.

Glucose, fructose and sucrose were assayed by high pH anion exchange chromatography with pulsed amperometric detection using a CarboPac PA-100 column following the manufacturer's protocol (Dionex) in samples of tissue from "first generation" transgenic tubers (tubers stored for three months at 4°C) (the antisense plants from Example 3). Compared with transgenic plants transformed with only the vector (pBIN19), a decrease in sucrose content was measured in some transgenic *MAL1* antisense lines (table 2 below). In the line that exhibited the strongest effect, sucrose levels were approximately 30 to 60% of the control values.

Table 2. Levels of glucose, fructose and sucrose in stored potato tubers from *MAL1* antisense plants compared with those from vector-only transformed plants.

(Data from selected lines to show range of values

obtained in antisense plants, values for antisense plants mean of 5 replicates from different tubers of the same transgenic line or mean of 8 replicates for the vector-only transformed control.)

Transgenic line	glucose mg glu/gfr.wt.	fructose mg fru/gfr.wt.	sucrose mg glu/gfr.wt.
2M1	0.05	0.05	1.08
3M4	0.09	0.08	1.74
11E	0.19	0.28	0.84
12C	0.05	0.08	1.17
12D	0.10	0.13	1.10
control	0.08	0.11	1.89

Example 6

Leaf starch turnover, maltose content and α-glucosidase activity.

The rate of starch turnover in fully expanded leaves from selected lines of antisense *MAL1* plants (from Example 3) was measured. Plants were grown in a growth cabinet at saturating light intensities with an 18 hour light period and a six hour dark period. Starch was extracted from leaves (Leidreiter *et al.* 1995) at the end of the light period and after four hours of darkness. The ratio of starch content in the light and dark was found to be consistently higher in the vector-only transformed lines (mean value 3.3) compared with the *MAL1* antisense lines (lowest values 2.4). The maltose content after 4 hours of darkness was also measured in leaf samples. Leaves from the *MAL1* antisense lines had up to twice the maltose content of those from vector-only transformed control lines. The results are shown in Table 3.

Table 3.

Starch content, α-glucosidase activity and maltose levels in leaves from *MAL1* antisense plants and vector-only transformed plants (control) after 18 hours light treatment (L) and 4 hours dark treatment (D). Data represent mean values of 5 samples.

Transgenic line	α-glucosidase activity (vector value = 100%)	maltose	starch content mM glu/gfr.wt		ratio(L/D)
			light	dark	
control	100	100	46.4	14.4	3.20
3M4	63	200	43.4	18.4	2.36
11E	54	204	50.2	19.2	2.60
14A	76	143	55.7	24.3	2.29

Example 7

Targetted expression of the MAL1 gene.

A construct has been been designed in which the expression of the *MAL1* gene is under the control of the tuber-specific GBSSI promoter (Visser *et al.*, 1989). The construct also contains the GBSSI targetting sequence so that the *MAL1* gene product was targetted to the amyloplast. Plants have been transformed with this construct and the *MAL1* gene product was expressed preferentially in the amyloplast.

Summary of the Transformation Studies.

Approximately 50 transgenic lines containing the *MAL1* sequence in sense and antisense orientation have been generated. In leaf samples of the antisense lines,

α-glucosidase activity was reduced by up to 50% compared with the vector control values. In tubers, few lines exhibited a consistent decrease in maltase activity and there was considerable tuber to tuber variability. Two consistent effects have been noticed however. Firstly, starch turnover rate (as determined by the ratio of light to dark starch content) was significantly lower in leaves from some *MAL1* antisense lines. Maltose levels in leaves from these antisense plants were higher (up to double that routinely measured in leaf samples from control plants). Secondly, in stored tubers from the antisense lines, the sucrose level was consistently lower than in control tubers, in some lines the sucrose content was only 30 to 60% of that observed in the control tubers.

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CLAIMS

1. Recombinant or isolated nucleic acid encoding an α-glucosidase enzyme, preferably a plant α-glucosidase enzyme.

2. Recombinant or isolated nucleic acid as claimed in claim 1 which is obtainable from potato.

3. Recombinant or isolated nucleic acid as claimed in claim 2 which is obtainable from the potato cultivar Record.

4. Recombinant or isolated nucleic acid, as claimed in any one of claims 1 to 3, wherein the enzyme has the activity of a 105.4 kD α-glucosidase enzyme of potato.

5. Recombinant or isolated nucleic acid as claimed in any one of claims 1 to 4 encoding at least part of the amino acid sequence as shown in Figure 2.

6. Recombinant or isolated nucleic acid as claimed in any one of claims 1 to 5, wherein a coding sequence is operatively linked to a promoter.

7. Recombinant or isolated nucleic acid comprising a promoter which naturally drives expression of a nucleic acid sequence encoding a plant α-glucosidase enzyme.

8. Recombinant or isolated nucleic acid as claimed in claim 7, wherein the coding nucleic acid sequence is as claimed in any one of claims 1 to 7.

9. Recombinant or isolated nucleic acid as claimed in any claim 6 to 8 which, when expressed, results in

enhanced starch and/or other carbohydrate breakdown in a plant or microbe.

10. Recombinant or isolated nucleic acid as claimed in any one of claims 6 to 9 including a signal sequence in translational fusion with the α-glucosidase coding sequence

11. Antisense nucleic acid which comprises a transcribable strand of nucleic acid, which is complementary to at least part of the strand of nucleic acid which is transcribed from a nucleic acid sequence encoding a α-glucosidase enzyme, preferably a plant α-glucosidase enzyme.

12. Antisense nucleic acid as claimed in claim 11 wherein the sequence encoding the α-glucosidase enzyme is obtainable from potato.

13. Antisense nucleic acid as claimed in claim 12, wherein the sequence encoding the α-glucosidase enzyme is obtainable from the potato cultivar Record.

14. Antisense nucleic acid as claimed in claim 13, wherein the α-glucosidase enzyme has the activity of a 105.4 kD α-glucosidase enzyme of potato.

15. Antisense nucleic acid as claimed in any one of claims 11 to 14 which is complementary to at least part of the nucleic acid sequence encoding the amino acid sequence as shown in Figure 2.

16. Antisense nucleic acid as claimed in any one of claims 11 to 15 operatively linked to a promoter.

17. Nucleic acid capable of specifically disrupting the proper expression of an α-glucosidase gene, preferably a plant α-glucosidase gene.

18. Nucleic acid as claimed in claim 17, wherein the α-glucosidase gene encodes a 105.4 kD α-glucosidase enzyme of potato.

19. Nucleic acid as claimed in any one of claims 1 to 18 comprising a 5' transcription regulation sequence.

20. Nucleic acid as claimed in claim 19, wherein the 5' transcription regulation sequence is derived from the Cauliflower Mosaic Virus 35S gene.

21. Nucleic acid as claimed in any one of claims 1 to 20 which is recombinant and comprises part of a vector.

22. Nucleic acid as claimed in claim 21, wherein the vector is a cloning or an expression vector and comprises one or more selectable markers.

23. A host cell transfected or transformed with a vector as claimed in claim 21 or claim 22.

24. A plant or microbial cell comprising nucleic acid as claimed in any one of claims 1 to 10 or 19 to 23 (when dependent on any one of claims 1 to 10).

25. A plant or microbial cell comprising nucleic acid as claimed in any one of claims 11 to 18 or 19 to 23 (when dependent on any one of claims 11 to 18).

26. A plant, microbe, or part of a plant, at least some

of whose cells are as claimed in claim 24.

27. A plant, microbe, or part of a plant, at least some of whose cells are as claimed in claim 25.

28. Use of nucleic acid as claimed in any one of claims 1 to 22 to modify starch and/or other carbohydrate breakdown in plant or microbial material.

29. Use of nucleic acid as claimed in any one of claims 1 to 10 or 19 to 23 (when dependent on any one of claims 1 to 10) to obtain enhanced α-glucosidase activity in plant or microbial material.

30. Use of nucleic acid as claimed in claim 29 to enhance flavour characteristics of plant tissue.

31. Use of a plant, or part of a plant, as claimed in claim 24 in a malting or brewing process.

32. Use of nucleic acid as claimed in any one of claims 11 to 18 or 19 to 23 (when dependent on any one of claims 11 to 18) to obtain reduced α-glucosidase activity in plant or microbial material.

33. Use of nucleic acid as claimed in claim 32 to control pre-sprouting in cereals, sprout control in potato or loss of starch and/or dry weight in storage, cooking or processing of plant material.

34. A novel starch structure obtainable by expression of nucleic acid as claimed in any one of claims 1 to 22.

```
   1  CGACGGCCAGTGAATTCCGGTCTTCTATGATATCTTTCCACATTGATCTCCGATTATAAC   60
  61  CATGAGAGCTCCACTACTCCTATATCCACTCCTTCTCCTCCTTCTATTTGTTACCTCTGC  120
 121  CTACTCCTGGAAGAAGGAGGAGTTTCGAAACTGCGACCAAACCCCATTTTGCAAAAGAGC  180
 181  CCGTTCCCGAAAACCTGGATCGTGCAATCTGCGGGTTGCCGATGTGTCCATCTCCGATGG  240
 241  GGATCTTATAGCCAAACTTGTCCCCAAAGAAGAAAACCCAGAAAGTGAACAACCCAATAA  300
 301  GCCTTTGGTTCTCACTCTTTCTGTGTACCAAGATGGTGTGATGAGGGTGAAAATTGATGA  360
 361  AGATCAAAATCTGAATCCACCCAAGAAAAGATTTGAAGTTCCTGAGGTGATTGAGGAAGA  420
 421  TTTCCTCAACACCAAGCTGTGGTTAACCAGAGTAAAAGAGGAGCAAATCGACGGAGTTTC  480
 481  GAGTTTTTCCTCTGTTTTTTACTTGTCTGATGGGTATGAAGGGGTGTTGAGACATGACCC  540
 541  ATTTGAGGTTTTTGCCAGAGAGAGTGGAAGTGGGAAGAGAGTGTTGTCCATAAACTCAAA  600
 601  TGGGTTGTTTGATTTTGAACAGTTGAGGGAGAAGAAAGAAGGGGATGATTGGGAGGAGAA  660
 661  GTTTAGGAGTCATACTGATACAAGGCCTTATGGTCCACAATCAATTAGTTTTGATGTATC  720
 721  TTTTTATGGTGCAGATTTTGTTTATGGCATTCCTGAACATGCTACTAGTTTTGCTTTGAA  780
 781  ACCAACTAAGGGGCCTAATGTAGAGGAATATTCCGAGCCTTATAGGTTATTTAATCTTGA  840
 841  TGTGTTTGAGTATCTTCATGAGTCGCCTTTTGGGCTTTATGGTTCAATTCCTTTCATGAT  900
 901  TTCACATGGGAAAGCCAGGGGTAGTTCGGGTTTTTTCTGGTTGAATGCTGCGGAAATGCA  960
 961  GATTGATGTATTGGGATCTGGTTGGAATTCAGATGAGTCTTCAAAGATAATGTTGCCCTC 1020
1021  GGACAAGCACAGGATTGATACTTTATGGATGAGTGAGTCTGGTGTAGTGGATACGTTCTT 1080
1081  TTTCATTGGTCCTGGGCCAAAAGATGTGGTTAGGCAGTATACTAGTGTAACGGGAAGGCC 1140
1141  ATCTATGCCACAGTTATTCGCAACTGCATACCATCAATGTAGATGGAATTATAGAGACGA 1200
1201  GGAAGATGTTTATAATGTTGATTCAAAATTTGATGAGCATGATATCCCTTATGATGTTTT 1260
1261  GTGGCTTGATATTGAGCACACAGATGGGAAGAAGTACTTTACTTGGGACAGGGTGTTATT 1320
1321  TCCTAACCCGGAAGAAATGCAGAAGAAGTTAGCTGCAAAGGGTAGACACATGGTTACCAT 1380
1381  TGTGGATCCTCATATCAAGAGGGATGAGTCTTACCATATACCCAAGGAGGCCTTAGAAAA 1440
1441  GGGATACTATGTTAAGGATGCTACTGGTAAGGATTATGATGGATGGTGCTGGCCTGGTTC 1500
1501  CTCATCATATACTGACTTGCTGAATCCCGAGATTAAGTCATGGTGGAGTGACAAATTTTC 1560
1561  ACTTGATAGCTATGTTGGCTCAACAAAGTATTTATACATCTGGAATGACATGAATGAACC 1620
1621  TTCCGTCTTCAATGGACCAGAGGTAACAATGCCAAGAGATGCTTTACATCATGGAGGAGT 1680
1681  AGAGCACAGGGAGTTGCACAATTCATATGGTTACTATTTCCATATGGGAACATCCGACGG 1740
1741  GCTTCTAAAGCGTGGAGATGGAAAAGATAGGCCTTTTGTTTTGGCAAGGGCCTTCTTTGC 1800
1801  CGGAAGTCAAAGATATGGAGCAATTTGGACTGGAGATAATACAGCAGAATGGGAGCACTT 1860
1861  GAGGGTTTCAGTCCCCATGGTGTTAACTCTTAGCATCTCTGGAATAGTATTTTCTGGTGC 1920
1921  AGATGTTGGTGGATTTTTTGGTAATCCTGACACTGAGTTGTTGGTTCGCTGGTATCAAGT 1980
1981  AGGTGCATATTATCCCTTTTTCCGGGGGCATGCACATCATGACACTAAAAGACGGGAACC 2040
2041  TTGGTTATTTGGAGAAAGAAATACACAATTGATGAGGGAAGCGATACATGTTCGTTACAT 2100
2101  GTATCTTCCTTATTTCTACACTCTATTTAGAGAAGCAAATTCAAGTGGTACTCCAGTTGC 2160
2161  TCGCCCACTTTGGATGGAGTTCCCTGGAGACGAAAAATCTTTTAGCAATGATGAGGCTTT 2220
2221  CATGGTTGGGAATGGTCTTCTAGTGCAAGGAGTTTATACAGAGAAACCAAAACATGTTTC 2280
2281  TGTCTATCTACCAGGGGAGGAATCCTGGTATGATTTAAGAAGTGCATCTGCATACAACGG 2340
2341  AGGTCATACACACAAGTATGAGGTTTCAGAAGATAGTATTCCTTCTTTTCAAAGGGCCGG 2400
2401  AACTATCATACCAAGGAAAGATCGTTTACGTCGGAGCTCGACACAGATGGAAAATGATCC 2460
2461  TTATACTCTGGTTATAGCTCTTAATAGTTCCAAGGCAGCTGAAGGTGAGCTTTATATCGA 2520
2521  TGATGGGAAGAGCTATGAGTTCAAACAAGGTGCCTTCATTCTCAAATGGGAGGCTTATAT 2580
2581  CTTCCAAATGCAGCCCCGTCTACAGCTGGCAGTGACACATTTTCCTTCCGAGTGCACTGT 2640
2641  AGAGAGGATAATCTTGTTAGGATTGTCTCCTGGAGCTAAAACAGCCCTTATTGAACCAGG 2700
2701  AAACAAGAAAGTTGAAATTGAGCTTGGGCCACTCTTCATTCAAGGAAATCGAGGATCTGT 2760
2761  TCCAACCATCCGCAAGCCTAATGTGCGTATTACAGATGATTGGTCGATTCAAATTTTGTA 2820
2821  AGAAGTTGGTAGTTATGACGAAGTCTTACATTTTCCTCTTCCGCGTTCATCATGTATTGG 2880
2881  GTTAGACCAGCTAATAGGTAAATCTTTACTAGAAGAATTAAACATTGATGAAGATTGTTA 2940
2941  TACAGGGATAGTTTGAAGCGGCCGCGAATTCGAGCTCGGTACCCGGGGATCC  2992
```

FIG. 1.

```
  1  MRAPLLLYPL LLLLLFVTSA YSWKKEEFRN CDQTPFCKRA RSRKPGSCNL
 51  RVADVSISDG DLIAKLVPKE ENPESEQPNK PLVLTLSVYQ DGVMRVKIDE
101  DQNLNPPKKR FEVPEVIEED FLNTKLWLTR VKEEQIDGVS SFSSVFYLSD
151  GYEGVLRHDP FEVFARESGS GKRVLSINSN GLFDFEQLRE KKEGDDWEEK
201  FRSHTDTRPY GPQSISFDVS FYGADFVYGI PEHATSFALK PTKGPNVEEY
251  SEPYRLFNLD VFEYLHESPF GLYGSIPFMI SHGKARGSSG FFWLNAAEMQ
301  IDVLGSGWNS DESSKIMLPS DKHRIDTLWM SESGVVDTFF FIGPGPKDVV
351  RQYTSVTGRP SMPQLFATAY HQCRWNYRDE EDVYNVDSKF DEHDIPYDVL
401  WLDIEHTDGK KYFTWDRVLF PNPEEMQKKL AAKGRHMVTI VDPHIKRDES
451  YHIPKEALEK GYYVKDATGK DYDGWCWPGS SSYTDLLNPE IKSWWSDKFS
501  LDSYVGSTKY LYIWNDMNEP SVFNGPEVTM PRDALHHGGV EHRELHNSYG
551  YYFHMGTSDG LLKRGDGKDR PFVLARAFFA GSQRYGAIWT GDNTAEWEHL
601  RVSVPMVLTL SISGIVFSGA DVGGFFGNPD TELLVRWYQV GAYYPFFRGH
651  AHHDTKRREP WLFGERNTQL MREAIHVRYM YLPYFYTLFR EANSSGTPVA
701  RPLWMEFPGD EKSFSNDEAF MVGNGLLVQG VYTEKPKHVS VYLPGEESWY
751  DLRSASAYNG GHTHKYEVSE DSIPSFQRAG TIIPRKDRLR RSSTQMENDP
801  YTLVIALNSS KAAEGELYID DGKSYEFKQG AFILKWEAYI FQMQPRLQLA
851  VTHFPSECTV ERIILLGLSP GAKTALIEPG NKKVEIELGP LFIQGNRGSV
901  PTIRKPNVRI TDDWSIQIL
```

FIG. 2.

```
pot    1 .....MRAPLLLYPLLLLLLFVT...SAYSWKKE 25
hum    1 MGVRHPPCSHRLLAVCALVSLATAALLGHILLHDFLLVPRELSGSSPVLE 50
pot   27 EFRNCDQTPFCKRARSRKPGSCNLRVADVSISDGDLIAKLVPKEENPESE 76
hum   51 ETHPAHQQGASRPGPRDAQAHPGRPRAVPTQCDVPPNSRFDCAPDKAITQ 100
pot   77 QPNKPLVLTLSVYQDGVMRVKIDEDQNLNP...PKKRFEVPEVIEEDFLN 123
hum  101 EQCEARGCCYIPAKQGLQGAQMGQPWCFFPPSYPSYKLENLSSSEMGYTA 150
pot  124 TKLWLTR......VKEEQIDGVSSFSSVFYLSDGYEGVLRHD.PFEVFAR 166
hum  151 TLTRTTPTFFPKDILTLRLDVMMETENRLHFTIKDPANRRYEVPLETPRV 200
pot  167 ESGSGKRVLSIN.SNGLFDFEQLREKKEGDDWEEKFRSHTDTRP..YGPQ 213
hum  201 HSRAPSPLYSVEFSEEPFGVIVHR......QLDGRVLLNTTVAPLFFADQ 244
pot  214 SISFDVSFYGADFVYGIPEHATSFALKPTKGPNVEEYSEPYRLFNLDVFE 263
hum  245 FLQLSTSL.PSQYITGLAEHLSPLMLSTS........WTRITLWNRDL.. 283
pot  264 YLHESPFGLYGSIPFMISHGKARGSSGFFWLNAAEMQIDVLGSGWNSDES 313
hum  284 .APTPGANLYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALS... 329
pot  314 SKIMLPSDKHRIDTLWMSESGVVDTFFFIGPGPKDVVRQYTSVTGRPSMP 363
hum  330 ..............WRSTGGILDVYIFLGPEPKSVVQQYLDVVGYPFMP 364
pot  364 QLFATAYHQCRWNYRDEEDVYNVDSKFDEHDIPYDVLWLDIEHTDGKKYF 413
hum  365 PYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQWNDLDYMDSRRDF 414
pot  414 TWDRVLFPNPEEMQKKLAAKGRHMVTIVDPHIK...RDESYHIPKEALEK 460
hum  415 TFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISSSGPAGSYRPYDEGLRR 464
pot  461 GYYVKDATGKDYDGWCWPGSSSYTDLLNPEIKSWWSDKFSLDSYVGSTKY 510
hum  465 GVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDM..VAEFHDQVPF 512
pot  511 LYIWNDMNEPSVF.NGPEVTMPRDALHHGG..................... 539
hum  513 DGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSH 562
```

FIG. 3.

```
pot  540 ...VEHRELHNSYGYYFHMGTSDGLLKRGDGKDRPFVLARAFFAGSQRYG 586
hum  563 QFLSTHYNLHNLYGLTEAIASHRALVKARGT..RPFVISRSTFAGHGRYA 610

pot  587 AIWTGDNTAEWEHLRVSVPMVLTLSISGIVFSGADVGGFFGNPDTELLVR 636
hum  611 GHWTGDVWSSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVR 660

pot  637 WYQVGAYYPFFRGHAHHDTKRREPWLFGERNTQLMREAIHVRYMYLPYFY 686
hum  661 WTQLGAFYPFMRNHNSLLSLPQEPYSFSEPAQQAMRKALTLRYALLPHLY 710

pot  687 TLFREANSSGTPVARPLWMEFPGDEKSFSNDEAFMVGNGLLVQGVYTEKP 736
hum  711 TLFHQAHVAGETVARPLFLEFPKDSSTWTVDHQLLWGEALLITPVLQAGK 760

pot  737 KHVSVYLPGEESWYDL.................RSASAYNGGHTHKYEV 768
hum  761 AEVTGYFP.LGTWYDLQTVPIEALGSLPPPPAAPREPAIHSEGQWVTLPA 809

pot  769 SEDSIPSFQRAGTIIPRKDRLRRSSTQMENDPYTLVIALNSSKAAEGELY 818
hum  810 PLDTINVHLRAGYIIPLQGP.GLTTTESRQQPMALAVALTKGGEARGELF 858

pot  819 IDDGKSYEFKQGAFILKWEAYIFQMQPRLQLAVTHFPSECTVERIILLGL 868
hum  859 WDDGESLE......VLERGAY.....................TQVIFLAR 881

pot  869 SPGAKTALIEPGNKKVEIELGPLFIQGNRGSVPTIRKPNVRITDDWSIQI 918
hum  882 NNTIVNELVRVTSEGAGLQLQKVTVLG....VATAPQ.....QV 916

pot  919 L*
hum  917 LSNGVPVSNFTYSPDTKVLDICVSLLMGEQFLVSWC..... 952
```

FIG. 3 contd.

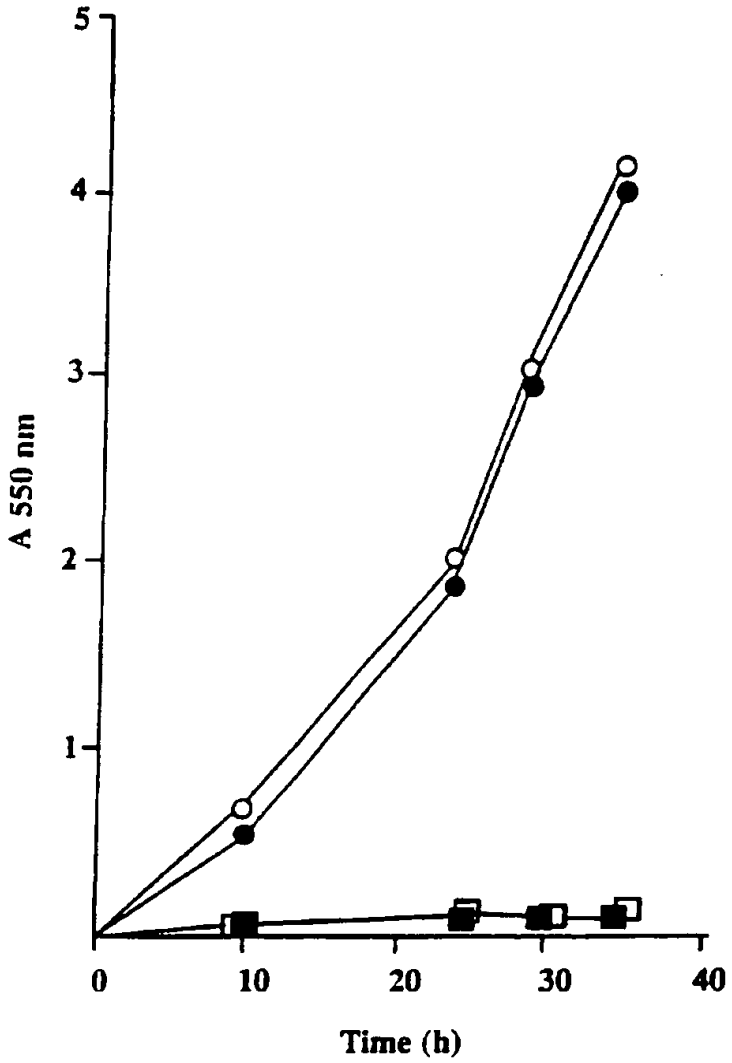

FIG. 4.

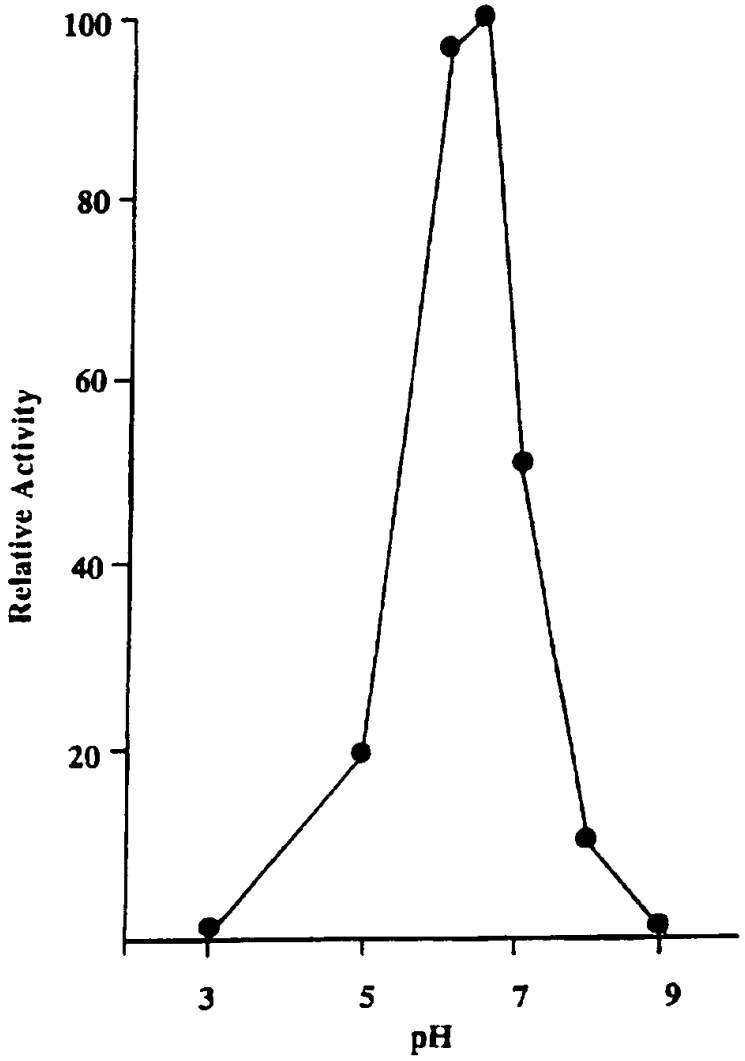

FIG. 5.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/03239

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/55 C12N1/21 C12N1/18 C12N5/10
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 42 13 444 A (INST GENBIOLOGISCHE FORSCHUNG) 28 October 1993 see column 3, line 9 - line 41; claims 1-3	1,6,11, 16,17, 19, 21-28, 32-34
X	EMBL SEQUENCE DATABASE, REL. 44, 17-AUG-1995, ACCESSION NO. U22450, XP002029013 TIBBOT, B.K., ET AL.: "Hordeum vulgare alpha-glucosidase mRNA, complete cds." see sequence	1,5,9, 11,15, 17,21-25
X	GB 2 247 238 A (GUINNESS SON & CO LTD A) 26 February 1992 see the whole document	1,5,6, 9-11,19, 21-29

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
8 April 1997	14.04.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intc. nal Application No
PCT/GB 96/03239

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 260 404 A (HEINEKEN TECHNISCHE BEHEER BV) 23 March 1988 see the whole document	1,5,6, 9-11,19, 21-29
X	THE EMBO JOURNAL, vol. 7, no. 6, 1988, pages 1697-1704, XP002029014 HOEFSLOOT, L.H., ET AL.: "Primary structure and processing of lysosomal alpha-glucosidase; homology with the intestinal sucrase-isomaltase complex" cited in the application see the whole document	1
X	EMBL SEQUENCE DATABASE, REL.36, 30-AUG-1993, ACCESSION NO. T04464, XP002029015 NEWMAN, T., ET AL.: "511 Arabidopsis thaliana cDNA clone 35C12T7" cited in the application see sequence	5
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